Immunity
Article

Plasmacytoid Dendritic Cells Are Crucial for the Initiation of Inflammation and T Cell Immunity In Vivo

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SUMMARY

Plasmacytoid dendritic cells (pDCs) are characterized as type I interferon-producing cells that engage endosomal toll-like receptors (TLRs) and exclusively express sialic acid binding Ig-like lectin (Siglec)-H. However, their role in vivo remains unclear. Here we report a critical role for pDCs in the regulation of inflammation and T cell immunity in vivo by using gene-targeted mice with a deficiency of Siglec-H and conditional ablation of pDCs. pDCs were required for inflammation triggered by a TLR ligand as well as by bacterial and viral infections. pDCs controlled homeostasis of effector and regulatory $CD4^+$ T cells. Upon antigenic stimulation and microbial infection, pDCs suppressed the induction of $CD4^+$ T cell responses and participated in the initiation of $CD8^+$ T cell responses. Furthermore, Siglec-H appeared to modulate the function of pDCs in vivo. Thus, our findings highlight previously unidentified roles of pDCs and the regulation of their function for the control of innate and adaptive immunity.

INTRODUCTION

Plasmacytoid dendritic cells (pDCs), referred to as natural interferon (IFN)-producing cells (IPCs), were originally discovered in humans as a small subset of blood leukocytes specialized in the secretion of high amounts of type I IFN in response to viruses, and the murine counterparts of human IPCs were identified on the basis of their phenotypic and functional characteristics (Gilliet et al., 2008; Swiecki and Colonna, 2010). In contrast to conventional DCs (cDCs), pDCs uniquely express toll-like receptor (TLR) 7 and 9, intracellular receptors that recognize single-stranded RNA or unmethylated CpG DNA within endosomal compartments (Gilliet et al., 2008; Swiecki and Colonna, 2010). Upon binding to their ligands, both TLRs recruit a cytoplasmic adaptor myeloid differentiation factor 88 (MyD88) and initiate downstream signaling pathways, in which the activation of the transcription factor nuclear factor κB (NF-κB) results in the production of proinflammatory cytokines, whereas the activation of inhibitor of NF-κB (IκB) kinase (IKK)-α leads to phosphorylation and nuclear translocation of IFN regulatory factor-7 (IRF-7), a critical event for the production of type I interferon (Hoshino et al., 2006).

pDCs are believed to provide an initial line of host defense against viral infection mainly mediated by the production of type I IFN (Gilliet et al., 2008; Swiecki and Colonna, 2010). In addition, pDCs may act as antigen-presenting cells (APCs) to exert a pleiotropic activating or inhibitory role in the regulation of T cell-mediated adaptive immune responses and immunopathogenesis (Salio et al., 2004; Ochando et al., 2006; Gilliet et al., 2008; Irla et al., 2010). However, whereas observations in vitro and ex vivo have suggested such functions, the precise role of pDCs in vivo remains unclear. One way to analyze pDCs in vivo is to delete them through treatment with monoclonal antibodies (mAbs) that recognize molecules on their surface, such as Gr-1 (Asselin-Paturel et al., 2001; Yoneyama et al., 2005) or bone marrow stromal antigen 2 (BST2) (Yoneyama et al., 2005; Kuwajima et al., 2006; Shen and Iwasaki, 2006; Goubier et al., 2008). However, this approach remains problematic because Gr-1 and BST2 are constitutively expressed on other immune cells or expressed on most cell types after activation, resulting in the depletion of additional cell types (Swiecki and Colonna, 2010). Therefore, the in vivo analysis of pDCs has been hampered by the lack of a system that selectively eliminates this cell subset during the inflammatory immune response.

To bypass such limitations, we used a gene targeting approach based on the diphtheria toxin receptor (DTR) (Jung et al., 2002; Kissenpfennig et al., 2005) and targeted the gene coding for sialic acid binding Ig-like lectin (Siglec)-H because it constitutes a pDC-specific functional molecule. The corresponding strategy allowed not only a deficiency of Siglec-H but also the specific

ablation of pDCs. With these mice, we obtained evidence that pDCs play a crucial role in the regulation of inflammation and T cell immunity in vivo and that Siglec-H controls pDCs function.

RESULTS

Deficiency of Siglec-H Modulates the Phenotype and Function of pDCs

The analysis of gene expression among immune cells showed that *Siglech* was predominantly expressed in pDCs but was not present in other leukocytes including cDC subsets (data not shown). Consistent with published reports (Blasius and Colonna, 2006; Zhang et al., 2006), the cell surface expression of Siglec-H was exclusively detected on BST2$^+$ pDCs among leukocytes in spleen (Spl), mesenteric lymph nodes (MLNs), and bone marrow (BM) under steady-state conditions (data not shown). Furthermore, BST2 was expressed on many cell types after stimulation with CpG-A (a TLR9 ligand) in vivo, whereas Siglec-H was expressed only on CD11c$^+$CD11b$^-$B220$^+$ subsets (data not shown).

To generate mice allowing specific elimination of pDCs in vivo, we designed a targeting construct in which a cDNA encoding the human DTR fused to sequences encoding enhanced green fluorescent protein (EGFP) and equipped with an internal ribosome entry site (IRES) (Kissenpfennig et al., 2005) was inserted into the 3′ untranslated region of the *Siglech* gene to produce *Siglech*$^{dtr/dtr}$ mice. *Siglech*$^{dtr/dtr}$ mice were born at the expected frequencies, and homozygous mice were healthy.

To address the influence of the introduction of the IRES-DTR-EGFP cassette into the *Siglech* locus, we compared the features of pDCs between wild-type (WT) mice and *Siglech*$^{dtr/dtr}$ mice. Unexpectedly, pDCs from *Siglech*$^{dtr/dtr}$ mice were deficient in the transcriptional and cell surface expression of Siglec-H (Figure 1A and Figure S1A available online), possibly owing to interference with the transcriptional or posttranscriptional machinery. Furthermore, pDCs from *Siglech*$^{dtr/dtr}$ mice displayed higher amounts of CD11c and BST2 and lower amounts of B220 and major histocompatibility complex class II (MHC II; I-A/I-E) molecules than those from WT mice (Figure 1A), indicating that Siglec-H affects the developmental process of pDCs in vivo.

We next examined TLR9-mediated cytokine production by pDCs. Siglec-H-deficient pDCs produced higher amounts of IFN-α and interleukin (IL)-12p40 than did WT pDCs (Figure 1B) although there was no substantial difference in the subcellular distribution of CpG-A-Cy5 and TLR9 in LAMP-1-positive late endosomes and lysosomes (Figure S1B). In contrast, cDCs from WT mice and *Siglech*$^{dtr/dtr}$ mice produced the same high amounts of IL-12p40 after stimulation with CpG-A, although they produced little or no IFN-α (Figure 1C).

We next investigated the signaling mechanism by which the lack of Siglec-H resulted in increased cytokine production in pDCs. Upon stimulation with CpG-A, there was no substantial difference in the amounts of phosphorylated ERK, JNK, and p38 between WT pDCs and Siglec-H-deficient pDCs (Figures S1C and S1D). The activation of NF-κB, indicated by the phosphorylation and degradation of IκBα (Kaisho and Tanaka, 2008), was enhanced in Siglec-H-deficient pDCs as compared with WT pDCs (Figures S1C and S1D). Furthermore, Siglec-H-deficient pDCs showed greater phosphorylation of IKK-α and the association of IKK-α with IRF-7 after stimulation with CpG-A than WT pDCs (Figures S1C and S1D). We also observed that a Siglec-H mAb (Blasius and Colonna, 2006) slightly and substantially suppressed the phosphorylation of IκBα and IKK-α, respectively, in WT pDCs after stimulation with CpG-A (Figure S1D). Therefore, the increased cytokine production observed in pDCs deficient in Siglec-H appears to be due to the enhanced TLR9-mediated activation of NF-κB and IKK-α.

Taken together, these results indicate that Siglec-H provides an inhibitory signal for TLR9-mediated downstream cascades to abrogate the cytokine production.

We determined next the influence of the deficiency of Siglec-H on the ability of pDCs to prime CD4$^+$ T cells through the MHC II-dependent presentation of exogenous antigens and CD8$^+$ T cells via the cross-presentation pathway, which is the capacity to deliver antigens to MHC class I (MHC I) molecules (Villadangos and Young, 2008). Accordingly, we compared the capacity of cDCs and pDCs from WT mice and *Siglech*$^{dtr/dtr}$ mice to present soluble ovalbumin (OVA) protein or peptides via OVA-specific T cell receptor (TCR) transgenic OT-II CD4$^+$ T cells and OT-I CD8$^+$ T cells (Dudziak et al., 2007; Hildner et al., 2008). The ability of pDCs to present OVA protein and peptide for the activation of OT-II CD4$^+$ T cells and OT-I CD8$^+$ T cells was substantially lower than that of cDCs in WT mice and *Siglech*$^{dtr/dtr}$ mice (Figures 1D–1K) and was accompanied by lower amounts of MHC I and II, CD40, and B7 family molecules (data not shown). When compared with WT pDCs, Siglec-H-deficient pDCs showed a reduced capacity to present OVA protein and peptide for priming of OT-II CD4$^+$ T cells (Figures 1D and 1E) because of the reduced expression of MHC II, although they had little ability to present OVA protein for the activation of OT-I CD8$^+$ T cells, possibly because of the defective cross-presentation pathway (Figures 1F and 1G). There was no major difference in the ability of cDCs to prime OT-II CD4$^+$ T cells and OT-I CD8$^+$ T cells between WT mice and Siglech-H-deficient mice (Figures 1H–1K).

Collectively, these results indicate that Siglec-H regulates the ability of pDCs to prime CD4$^+$ T cells and CD8$^+$ T cells.

Inducible Ablation of pDCs in Mice

To validate the *Siglech*$^{dtr/dtr}$ mice in terms of DT-induced pDC elimination, *Siglech*$^{dtr/dtr}$ mice received a single injection of DT, and we monitored 2 days later the subsequent frequency and the absolute number of pDCs among leukocytes in Spl, MLNs, and BM. Treatment with DT at a dose of 1 μg per mouse almost completely ablated pDCs among leukocytes in Spl, MLNs, and BM in *Siglech*$^{dtr/dtr}$ mice, whereas this treatment had no effect in WT mice (Figures 2A and 2B). Furthermore, the efficacy of the ablation was greater in *Siglech*$^{dtr/dtr}$ mice than in *Siglech*$^{dtr/+}$ mice (Figure 2A). In contrast, there was no change in the proportion of cDCs and other leukocytes in Spls, MLNs, and BM as well as the content of thymocytes in *Siglech*$^{dtr/dtr}$ mice as well as WT mice after injection with DT (Figure 2C and data not shown). Such DT-mediated ablation of pDCs occurred in a dose-dependent manner (Figure 2D). Near-complete elimination was achieved 24 hr after the injection of DT in *Siglech*$^{dtr/dtr}$ mice, but cell numbers gradually rebounded thereafter and had fully recovered by day 10 (Figure 2E). Histological analysis of *Siglech*$^{dtr/dtr}$ mice confirmed that pDCs disappeared after the injection of DT (Figure S2A), but there was no effect on the localization of CD11c^{hi}

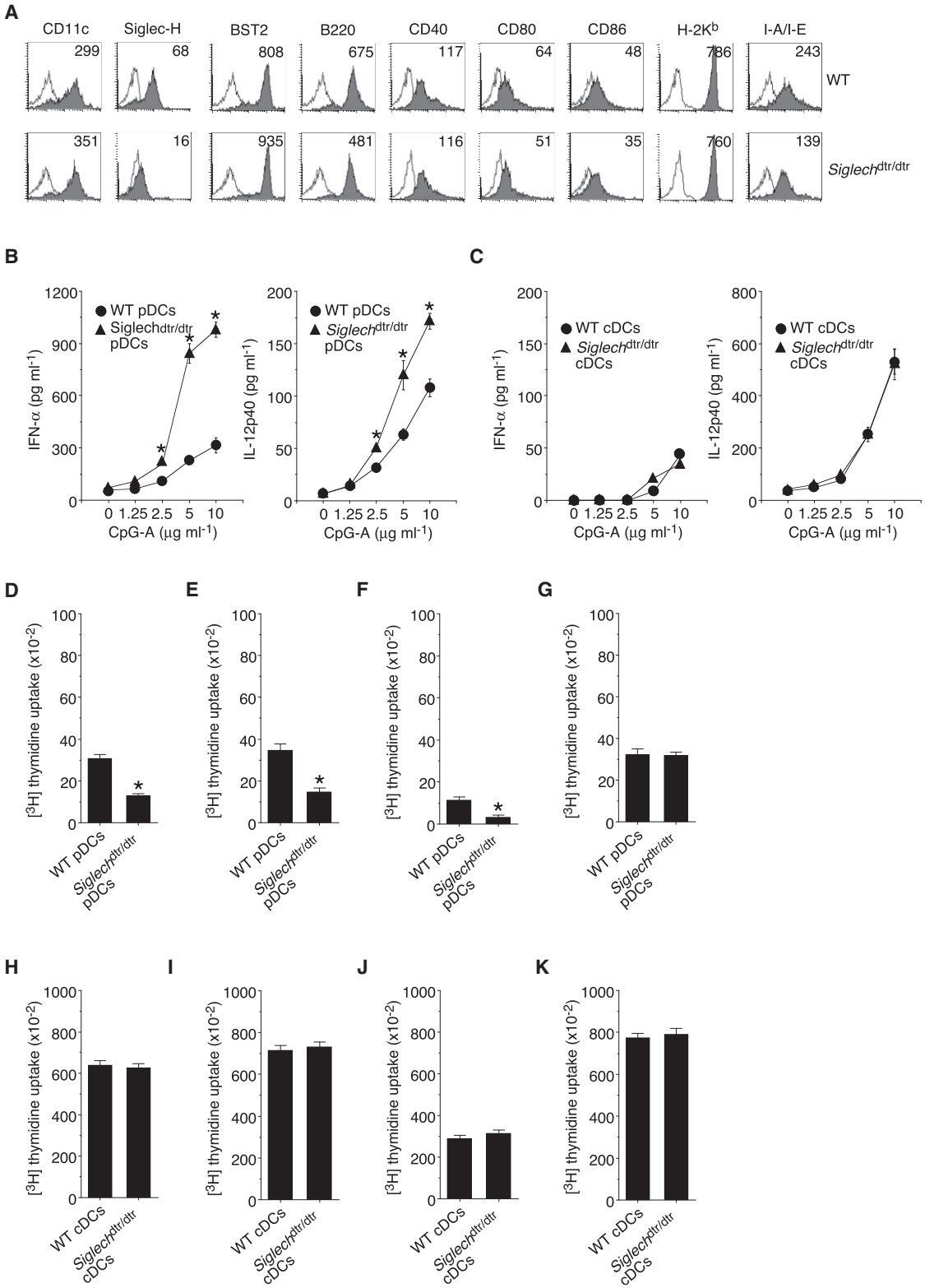

Figure 1. Deficiency of Siglec-H Modulates the Phenotype and Function of pDCs

(A) The expression of cell surface molecules on pDCs from WT mice and *Siglech*$^{dtr/dtr}$ mice was analyzed by flow cytometry. Data are represented by a histogram, and numbers represent mean fluorescence intensity (MFI).

(B and C) pDCs (B) and cDCs (C) from WT mice and *Siglech*$^{dtr/dtr}$ mice were stimulated or not stimulated with CpG-A, and the production of IFN-α (left) and IL-12p40 (right) was measured by enzyme-linked immunosorbent assay (ELISA).

cDCs (Figure S2B) or CD209b$^+$ marginal zone macrophages expressing intracellular Siglec-H (Figure S2C; Zhang et al., 2006).

Therefore, *Siglech*$^{dtr/dtr}$ mice provide the opportunity to analyze the effect of a deficency of Siglec-H or the effect of conditional ablation of pDCs in vivo, the two models referred to as Siglec-H-deficient mice and pDC-ablated mice, respectively.

Siglec-H and pDCs Control TLR-Mediated Inflammatory Responses In Vivo

We addressed the roles of Siglec-H and pDCs in the TLR-mediated inflammatory response in vivo. Administration of CpG-A caused serum production of IFN-α and IL-12p40 in WT mice (Figure 3A). Such production was markedly enhanced in Siglec-H-deficient mice, whereas pDC-ablated mice exhibited a dramatic reduction in serum cytokine amounts as compared with WT mice (Figure 3A).

We also examined lethal shock triggered by CpG-A in D-galactosamine (D-GalN)-sensitized mice. WT mice died within 24 hr after the administration of CpG-A plus D-GalN with a marked elevation in serum concentrations of IFN-α, IFN-β, tumor necrosis factor α (TNF-α), IL-6, and IL-12p40 (Figures 3B and 3C). In contrast, Siglec-H-deficient mice showed increased amounts of serum cytokines after the injection with CpG-A plus D-GalN, whereas pDC-ablated mice exhibited an enhanced survival rate ($p < 0.01$), which was accompanied by a substantial reduction in serum cytokine amounts (Figures 3B and 3C).

Taken together, these results indicate that pDCs initiate the TLR9-mediated inflammation in vivo and Siglec-H regulates their responsiveness to produce cytokines.

We further examined the TLR-mediated maturational changes of cDCs and pDCs in vivo. Whereas the administration of CpG-A increased the expression of MHC I (H-2K^b), MHC II, CD40, and B7 family molecules on cDCs and pDCs in WT mice, their amounts were further enhanced in Siglec-H-deficient mice (Figure S3). In addition, this treatment induced a more potent expression of CD154 on pDCs in Siglec-H-deficient mice than WT mice (Figure S3). In contrast, pDC-ablated mice exhibited lower amounts of MHC and costimulatory molecules on cDCs than WT mice (Figure S3).

Taken together, these results indicate that pDCs are involved in the TLR9-mediated optimal maturation of cDCs in vivo and that Siglec-H controls this process.

Siglec-H and pDCs Suppress Antigen-Specific CD4$^+$ T Cell Responses In Vivo

To determine the roles of Siglec-H and pDCs in antigen-specific CD4$^+$ T cell responses in vivo, carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled OT-II CD4$^+$ T cells were adoptively transferred into mice, and their division was analyzed 3 days after systemic injection of soluble OVA protein under steady-state conditions. Immunization with OVA protein resulted in antigen-specific division of OT-II CD4$^+$ T cells in WT mice (Figure 4A). When compared with WT mice, Siglec-H-deficient mice exhibited an enhancement of antigen-specific division of OT-II CD4$^+$ T cells, whereas pDC-ablated mice showed a vigorous response of OT-II CD4$^+$ T cells, and the reconstitution with WT pDCs restored this response (Figures 4A and S4).

We next examined antigen-specific CD4$^+$ T cell responses under inflammatory conditions. At 14 days after immunization with OVA protein emulsified in complete Freund's adjuvant (CFA), CD4$^+$ T cells from pDC-ablated mice showed more vigorous proliferation on restimulation with OVA protein than did CD4$^+$ T cells from Siglec-H-deficient mice, while both proliferated more than those from WT mice (Figure 4B). Alternatively, CD4$^+$ T cells from Siglec-H-deficient mice substantially reduced, whereas CD4$^+$ T cells from pDC-ablated mice markedly enhanced, the production of IFN-γ as well as the frequency of IFN-γ-producing cells (Th1 cells), as compared with the cells from WT mice (Figures 4C and 4D).

To assess the influence of the deficiency of Siglec-H and the ablation of pDCs on the antigen-specific differentiation of CD4$^+$ Foxp3$^-$ T cells into CD4$^+$Foxp3$^+$ inducible regulatory T (iTreg) cells (Fukaya et al., 2010) under steady-state and inflammatory conditions, we adoptively transferred OT-II CD4$^+$*Foxp3*$^{EGFP-}$ T cells into mice and monitored the generation of OT-II CD4$^+$ *Foxp3*$^{EGFP+}$ iTreg cells 8 days after systemic immunization with OVA protein (Figure 4E). In WT mice, immunization with OVA protein generated OT-II CD4$^+$*Foxp3*$^{EGFP+}$ iTreg cells from OT-II CD4$^+$*Foxp3*$^{EGFP-}$ T cells in steady-state conditions more than in inflammatory conditions. In contrast, the peripheral generation of OT-II CD4$^+$*Foxp3*$^{EGFP+}$ iTreg cells was reduced in Siglec-H-deficient mice compared with WT mice after systemic immunization, whereas it was severely diminished in pDC-ablated mice.

Collectively, these results indicate that pDCs inhibit antigen-specific CD4$^+$ T cell responses in vivo and that Siglec-H controls their ability to generate CD4$^+$ effector T (CD4$^+$ Teff) cells and CD4$^+$Foxp3$^+$ iTreg cells.

Siglec-H and pDCs Are Required for Antigen-Specific CD8$^+$ T Cell Responses In Vivo

To determine the contribution of pDCs and their Siglec-H-mediated regulation to the cross-presentation of soluble antigen for the activation of CD8$^+$ T cells in vivo, mice that had been adoptively transferred with CFSE-labeled OT-I CD8$^+$ T cells were immunized with OVA protein, and antigen-specific division was measured 3 days after immunization. In the steady state, WT mice showed antigen-specific division of OT-I CD8$^+$ T cells after immunization with OVA protein (Figure 5A). Alternatively, pDC-ablated mice and Siglec-H-deficient mice showed substantially less antigen-specific division of OT-I CD8$^+$ T cells than did WT mice, and in pDC-ablated mice, the reduction in cell division was overcome by reconstitution with WT pDCs (Figures 5A and S5).

Next we examined the impact of the deficiency of Siglec-H and ablation of pDCs on the generation of cytotoxic T lymphocytes (CTLs) through the cross-presentation of soluble antigen.

(D–K) CD45.1 OT-II CD4$^+$ T cells (D, E, H, I) or CD45.1 OT-I CD8$^+$ T cells (F, G, J, K) were cultured with pDCs (D–G) or cDCs (H–K) obtained from WT mice and *Siglech*$^{dtr/dtr}$ mice in the presence or absence of OVA protein (D, F, H, J), OVA$_{323-339}$ peptide (E, I), or OVA$_{257-264}$ peptide (G, K), and the proliferation was measured by [^{3}H]thymidine incorporation. The values for T cells alone and T cells plus DCs were less than 100 cpm.

*$p < 0.01$ compared with WT mice. Data are the mean ± SD, and the results are representative of four independent experiments with similar results. See also Figure S1.

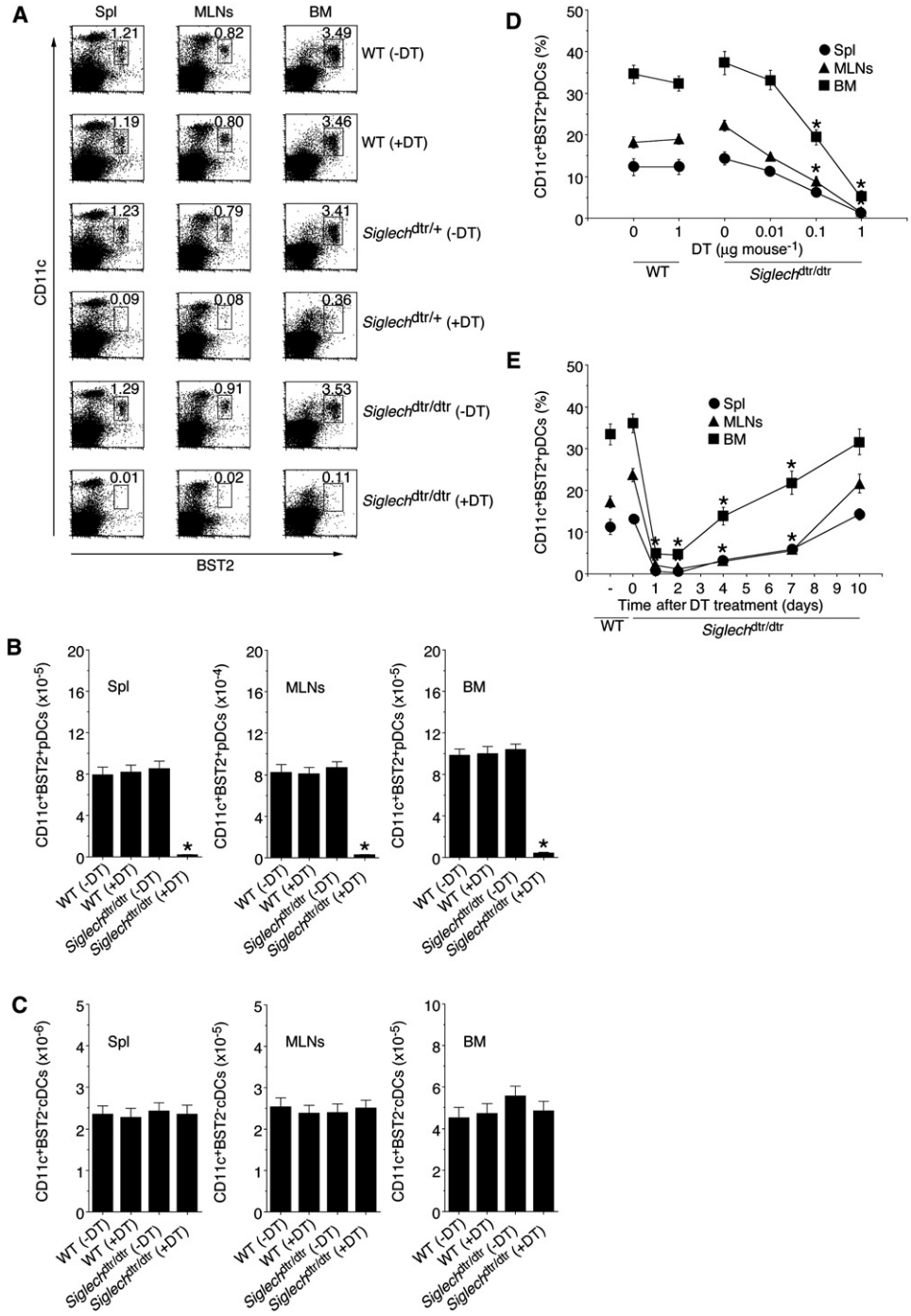

Figure 2. Conditional Ablation of pDCs in *Siglech*$^{dtr/dtr}$ Mice

(A–C) WT mice (n = 6), *Siglech*$^{dtr/+}$ mice (n = 6), and *Siglech*$^{dtr/dtr}$ mice (n = 6) were injected with DT (1 μg/mouse), and Spl, MLNs, and BM were obtained 2 days after the injection.

(A) The frequency of $CD11c^+BST2^+$ pDCs was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of $CD11c^+BST2^+$ cells among leukocytes in each quadrant.

(B and C) The absolute number of $CD11c^+BST2^+$ pDCs (B) and $CD11c^+BST2^-$ cDCs (C) was analyzed by flow cytometry.

(D) WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) were injected with various doses of DT, and Spl, MLNs, and BM were obtained 2 days after the injection. The frequency of $CD11c^+BST2^+$ pDCs was analyzed by flow cytometry. Data are the percentage of positive cells among $CD11c^+$ cells.

(E) WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) were injected with DT (1 μg/mouse), and Spl, MLNs, and BM were obtained at the indicated days after the injection. The frequency of $CD11c^+BST2^+$ pDCs was analyzed by flow cytometry.

Data are the percentage of positive cells among $CD11c^+$ cells. *$p < 0.01$ compared with WT mice. Data are the mean ± SD, and the results are representative of six independent experiments with similar results. See also Figure S2.

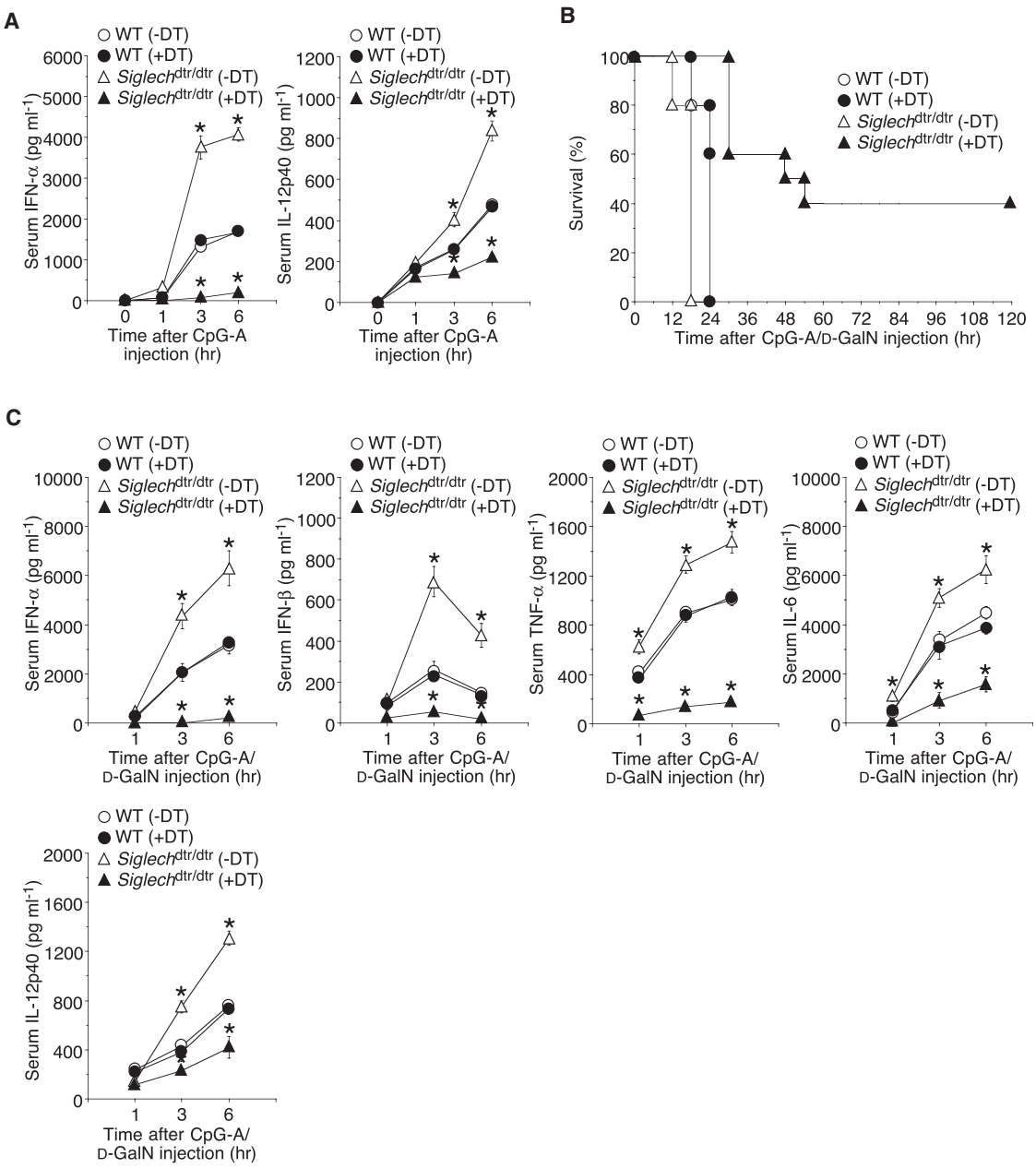

Figure 3. Deficiency of Siglec-H and Ablation of pDCs Affect TLR-Mediated Cytokine Production and Lethality

(A) WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse) were injected with CpG-A complexed to in vivo-jetPEI transfection reagent, and serum production of IFN-α (left) and IL-12p40 (right) was measured at the indicated time after injection by ELISA.

(B and C) WT mice (n = 10) and *Siglech*$^{dtr/dtr}$ mice (n = 10) that had been treated with DT (1 μg/mouse) were injected with CpG-A complexed to in vivo-jetPEI transfection reagent plus D-GalN.

(B) Survival was monitored for 5 days.

(C) Serum production of IFN-α, IFN-β, TNF-α, IL-6, and IL-12p40 was measured at the indicated time after injection by ELISA.

*$p < 0.01$ compared with WT mice. Data are the mean ± SD, and the results are representative of three independent experiments with similar results. See also Figure S3.

Mice were immunized with OVA protein combined with CpG-A and CD40 mAb, and we quantified the generation of antigen-specific $CD8^+$ T cells based on binding with the MHC I-OVA tetramer and intracellular expression of IFN-γ as well as their lytic activity against $OVA_{257-264}$ peptide-pulsed syngeneic splenocytes 6 days after immunization (Figures 5B–5D). WT mice showed efficient generation of MHC I-OVA tetramer$^+$CD44^{hi} $CD8^+$ T cells and $CD8^+$IFN-γ$^+$ T cells as well as substantial cytotoxic activity against targeted cells. In contrast, pDC-ablated mice had a more dramatic reduction in the generation

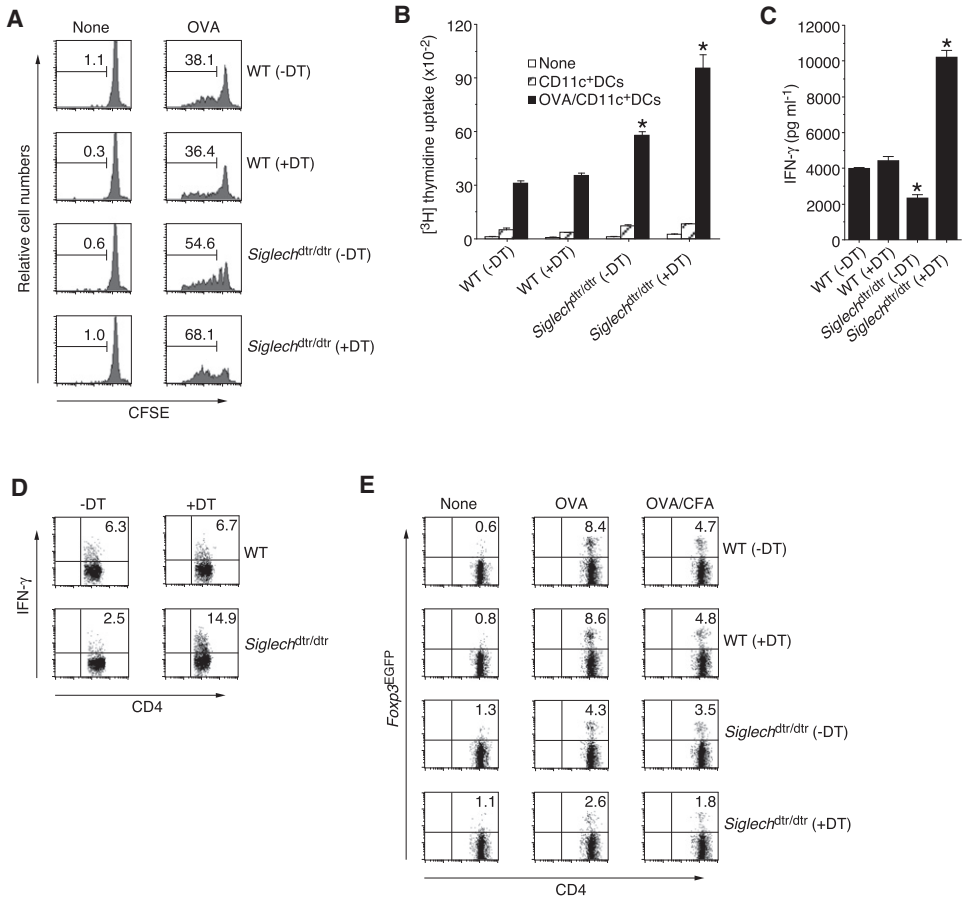

Figure 4. Deficiency of Siglec-H and Ablation of pDCs Affect Antigen-Specific CD4$^+$ T Cell Responses In Vivo

(A) CD45.1$^+$OT-II CD4$^+$ T cells were transferred into WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse), and then the mice were immunized with OVA protein. Antigen-specific division of CD45.1$^+$OT-II CD4$^+$ T cells was analyzed at 3 days after the immunization by flow cytometry. Data are represented by a histogram, and numbers represent the proportion of CFSE dilution among gated CD45.1$^+$OT-II CD4$^+$ T cells in each quadrant.

(B–D) WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse) were immunized with CFA plus OVA protein.

(B and C) At 14 days after the immunization, Spl CD4$^+$ T cells were cultured with WT CD11c$^+$ DCs in the presence or absence of OVA protein for the measurement of proliferative responses by [^{3}H]thymidine incorporation (B) and production of IFN-γ by ELISA (C).*$p < 0.01$ compared with WT mice. Data are the mean ± SD.

(D) Intracellular production of IFN-γ in the cultured CD4$^+$ T cells was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of IFN-γ$^+$ cells among gated CD4$^+$ T cells in each quadrant.

(E) CD45.1$^+$OT-II CD4$^+$*Foxp3*$^{EGFP-}$ T cells were transferred into WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse), and then the mice were immunized with OVA protein or CFA plus OVA protein. Expression of *Foxp3*^{EGFP} among gated CD45.1$^+$OT-II CD4$^+$ T cells in Spl was analyzed at 8 days after the immunization by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of *Foxp3*$^{EGFP+}$ cells among gated CD45.1 OT-II CD4$^+$ T cells in each quadrant.

The results are representative of three independent experiments with similar results. See also Figure S4.

of OVA-specific CTLs than did Siglec-H-deficient mice when compared with WT mice.

Taken together, these results indicate that pDCs are involved in the antigen-specific generation of CTLs in vivo, and Siglec-H regulates their capacity to cross presentation of soluble antigen.

Siglec-H and pDCs Control the Homeostasis of CD4$^+$Foxp3$^+$ Treg Cells and Th17 Cells In Vivo

To address the involvement of pDCs and their Siglec-H-mediated regulation in the homeostasis of CD4$^+$Foxp3$^+$ Treg cells, we analyzed the frequency and number of CD4$^+$Foxp3$^+$ Treg cells in Siglec-H-deficient mice and pDC-ablated mice (Figures 6A–6C). Both had a slightly higher frequency and number of thymic CD4$^+$Foxp3$^+$ naturally occurring regulatory T (nTreg) cells than did WT mice, whereas there was no difference in the population of splenic CD4$^+$Foxp3$^+$ nTreg cells. In addition, pDC-ablated mice, but not Siglec-H-deficient mice, displayed a substantial reduction in CD4$^+$Foxp3$^+$ nTreg cells in the lamina propria (LP) of the small intestine as compared with WT mice.

Previous studies have shown that IL-17-producing CD4$^+$ T cells (Th17 cells) constitute a considerable proportion of CD4$^+$ T cells in LP in contrast to other peripheral lymphoid tissues (Darrasse-Jèze et al., 2009; Ohnmacht et al., 2009). We therefore examined the roles of Siglec-H and pDCs in the homeostasis of Th17 cells as well as Th1 cells in LP of the small intestine (Figure 6D). Siglec-H-deficient mice showed a

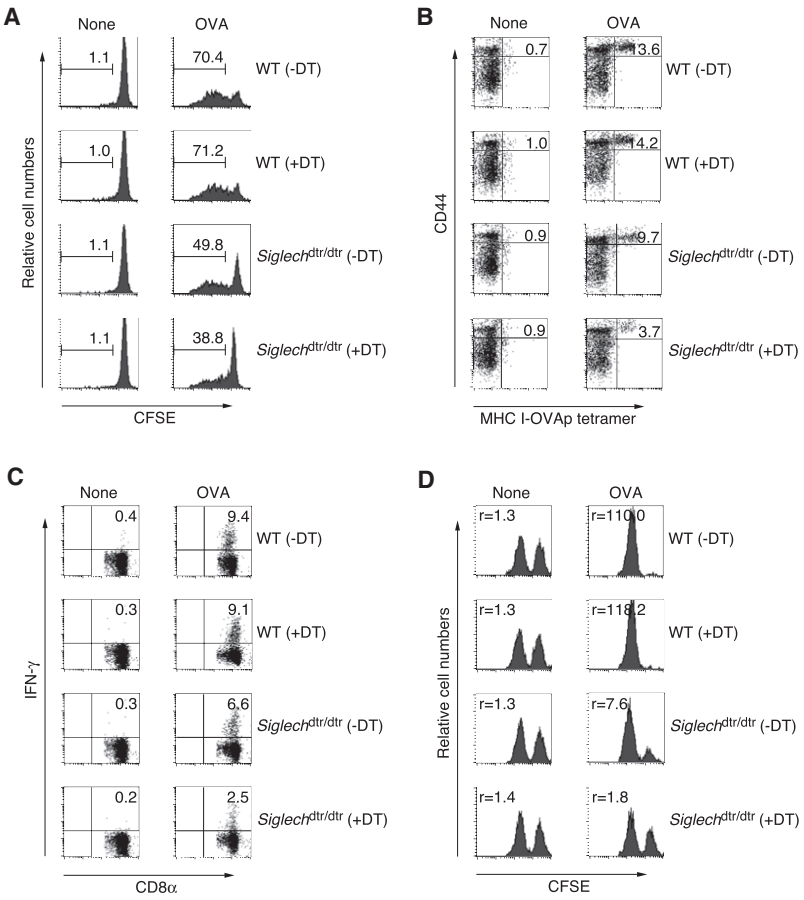

Figure 5. Deficiency of Siglec-H and Ablation of pDCs Affect Antigen-Specific CD8$^+$ T Cell Responses In Vivo

(A) CD45.1$^+$OT-I CD8$^+$ T cells were transferred into WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse), and then the mice were immunized with OVA protein. Antigen-specific division of CD45.1$^+$OT-I CD8$^+$ T cells was analyzed at 3 days after the immunization by flow cytometry. Data are represented by a histogram, and numbers represent the proportion of CFSE dilution among gated CD45.1$^+$OT-I CD8$^+$ T cells in each quadrant.

(B–D) WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse) were immunized with CpG-A complexed to in vivo-jetPEI transfection reagent, CD40 mAb, and OVA protein, and then a mixture of unpulsed CFSE^{lo} cells plus antigen-pulsed CFSE^{hi} cells was injected 5 days after the immunization. At 6 days after the immunization, splenocytes were analyzed for the generation of MHC I-OVA tetramer$^+$CD44^{hi}CD8$^+$ T cells (B), for intracellular IFN-γ-producing CD8$^+$ T cells (C), and for cytotoxic activity in vivo (D) by flow cytometry. Data are represented by a dot plot (B and C), and numbers represent the proportion of MHC I-OVA tetramer$^+$CD44^{hi} cells (B) and IFN-γ$^+$ cells (C) among gated CD8$^+$ T cells in each quadrant, or by a histogram (D), and numbers represent the ratio of unpulsed CFSE^{lo} cells to antigen-pulsed CFSE^{hi} cells.

The results are representative of three independent experiments with similar results. See also Figure S5.

substantially lower proportion and number of these CD4$^+$ Teff cells than did WT mice, whereas these populations were markedly enhanced in pDC-ablated mice.

Collectively, these results indicate that pDCs are required for the homeostasis of CD4$^+$Foxp3$^+$ Treg cells and CD4$^+$ Teff cells through the Siglec-H-mediated regulation in vivo.

Siglec-H and pDCs Control Host Defense against Bacterial and Viral Infections In Vivo

The role of pDCs in the regulation of the host immune response to bacterial infections in vivo is poorly understood. We therefore examined the influence of a deficiency of Siglec-H and the ablation of pDCs on inflammation caused by infection with *Listeria monocytogenes*-expressing OVA (LM-OVA). WT mice exhibited massive proinflammatory cytokine production 24 hr after infection with LM-OVA and died within 5 days of infection (Figures 7A and 7B), which was similar to microbial septic shock (Fujita et al., 2006). In contrast, pDC-ablated mice were resistant to lethal infection with LM-OVA with the decreased production of proinflammatory cytokines as compared with WT mice ($p < 0.01$; Figures 7A and 7B). Furthermore, they showed a higher absolute number of CD11c$^+$B220$^-$ cDCs and lower bacterial burden 3 days after infection with LM-OVA than WT mice (Figures 7C and 7D).

To assess antigen-specific T cell responses against LM-OVA in vivo, antigen-specific division of CFSE-labeled OT-II CD4$^+$ T cells or OT-I CD8$^+$ T cells transferred into mice was monitored 3 days after infection with LM-OVA. Infection of WT mice caused antigen-specific division of OT-II CD4$^+$ T cells and OT-I CD8$^+$ T cells (Figures 7E and 7F). pDC-ablated mice showed increased antigen-specific division of OT-II CD4$^+$ T cells more than did Siglec-H-deficient mice when compared with WT mice (Figure 7E). In contrast, pDC-ablated mice showed substantially less antigen-specific division of OT-I CD8$^+$ T cells than did WT mice, whereas such cell division was slightly reduced in Siglec-H-deficient mice (Figure 7F).

To clarify the role of pDCs and their Siglec-H-mediated regulation in protective immune responses against viral infections, we evaluated the production of serum cytokines and generation of CTLs after infection with herpes simplex virus-1 (HSV-1). Infection with HSV-1 triggered higher serum amounts of IFN-α and IL-12p40 in Siglec-H-deficient mice than in WT mice 6 hr after infection (Figure S6A). In contrast, the production of IFN-α was markedly reduced in pDC-ablated mice, whereas only a slight reduction in the production of IL-12p40 was observed compared with WT mice after infection with HSV-1 (Figure S6A). Alternatively, pDC-ablated mice exhibited a greater reduction in the generation of MHC I-HSV-1 gB tetramer$^+$CD44^{hi}CD8$^+$ T cells and CD8$^+$IFN-γ$^+$ T cells than in Siglec-H-deficient mice as compared with WT mice 6 days after infection (Figures S6B and S6C). Although we did not detect HSV-1 in Spl from WT mice 6 days after infection, pDC-ablated mice had a higher viral titer than did Siglech-H-deficient mice (Figure S6D).

Taken together, these results indicate that pDCs are involved in the initiation of inflammation and antigen-specific T cell

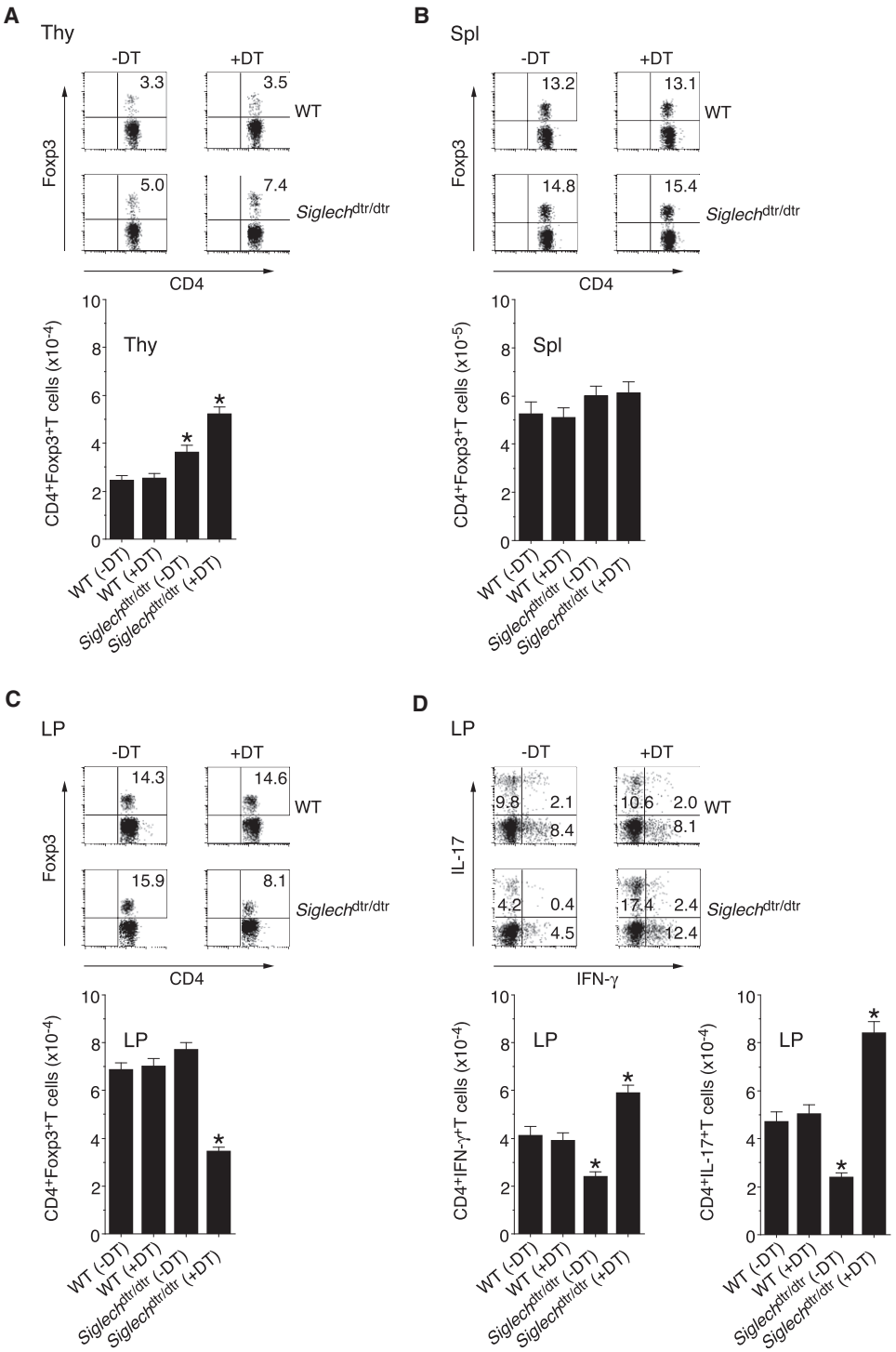

Figure 6. Deficiency of Siglec-H and Ablation of pDCs Affect the Homeostasis of CD4$^+$Foxp3$^+$ Treg Cells and Th17 Cells In Vivo

WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) were injected with DT (1 μg/mouse), and thymus (Thy), Spl, and LP were obtained 8 days after the injection. (A–C) The proportion of CD4$^+$Foxp3$^+$ Treg cells among gated CD4$^+$ T cells (top) and the absolute number (bottom) in Thy (A), Spl (B), and LP (C) was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of Foxp3$^+$ cells among gated CD4$^+$ T cells in each quadrant. (D) The proportion of intracellular IFN-γ- and IL-17-producing cells among gated CD4$^+$ T cells (top) and the absolute number (bottom) in LP was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of IFN-γ$^+$ cells and IL-17$^+$ cells among gated CD4$^+$ T cells in each quadrant. *$p < 0.01$ compared with WT mice. Data are the mean ± SD, and the results are representative of six independent experiments with similar results.

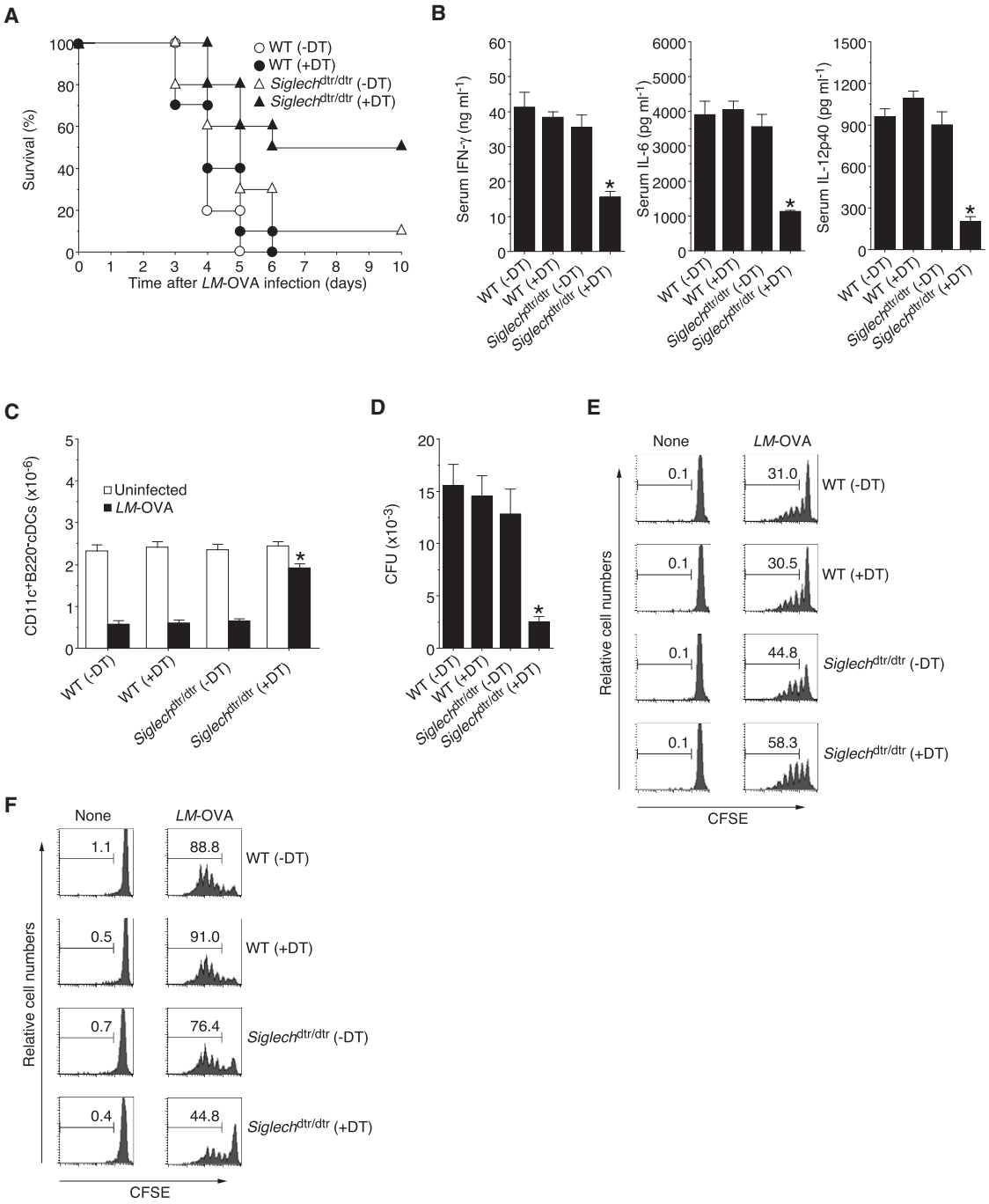

Figure 7. Deficiency of Siglec-H and Ablation of pDCs Affect Host Defenses against Bacterial Infection In Vivo

(A–D) WT mice (n = 10) and *Siglech*$^{dtr/dtr}$ mice (n = 10) that had been treated with DT (1 μg/mouse) were uninfected or infected with LM-OVA.

(A) Survival was monitored for 10 days.

(B) Serum production of IFN-γ, IL-6, and IL-12p40 was measured at 24 hr after infection by ELISA.

(C) The absolute number of CD11c^{+}B220^{-} cDCs was analyzed by flow cytometry 3 days after infection.

(D) Bacterial burden in the Spl was determined as colony-forming units (CFU) 3 days after infection.

*$p < 0.01$ compared with WT mice. Data are the mean ± SD.

(E and F) CD45.1^{+}OT-II CD4^{+} T cells (E) or CD45.1^{+}OT-I CD8^{+} T cells (F) were transferred into WT mice (n = 6) and *Siglech*$^{dtr/dtr}$ mice (n = 6) that had been treated with DT (1 μg/mouse), and then the mice were infected with LM-OVA. Antigen-specific division of CD45.1^{+}OT-II CD4^{+} T cells or CD45.1^{+}OT-I CD8^{+} T cells was analyzed at 3 days after infection by flow cytometry. Data are represented by a histogram, and numbers represent the proportion of CFSE dilution among gated CD45.1^{+}OT-II CD4^{+} T cells or CD45.1^{+}OT-I CD8^{+} T cells in each quadrant.

The results are representative of three independent experiments with similar results. See also Figure S6.

responses during microbial infections through the Siglec-H-mediated regulation.

DISCUSSION

In this paper, we describe a DTR-based gene targeting strategy that targets the *Siglech* gene and allows not only the effect of a deficiency of Siglec-H to be analyzed but also the selective elimination of pDCs in vivo. With those mice, we have demonstrated the importance of the functions of pDCs and their Siglec-H-mediated regulation for the control of innate and adaptive immune responses in vivo.

Whereas several cell types have been reported to respond to TLR9 ligand (Shen and Iwasaki, 2006), the contribution of pDCs to the TLR9-mediated response in vivo remains unclear. Administration of anti-BST2 severely abolished TLR9-mediated production of both IFN-α and IL-12 (Asselin-Paturel et al., 2003; Kuwajima et al., 2006). Because BST2 expression could be upregulated on the activated immune cells, this mAb could deplete not only pDCs but also other leukocytes producing these cytokines during inflammation (Swiecki and Colonna, 2010), resulting in a profound reduction of TLR9-mediated responses in vivo. Thus, pDCs could be the sole producer of type I IFN, while they might moderately contribute to proinflammatory cytokine release in response to TLR9 ligand, and Siglec-H directly controls their responsiveness in vivo.

The deficiency of Siglec-H enhanced the maturation of cDCs, whereas the ablation of pDCs reduced their changes after immunization. Therefore, the cross-talk between pDCs and cDCs could be important for the optimal activation of cDCs in vivo, possibly mediated through the pDC-derived cytokine production. Moreover, both the deficiency of Siglec-H and the ablation of pDCs enhanced antigen-specific $CD4^+$ T cell proliferation in vivo. Collectively, these results imply that pDCs promote the ability of cDCs to activate $CD4^+$ T cells, whereas pDCs themselves suppress antigen-specific $CD4^+$ T cell responses regardless of environmental conditions.

There has been some controversy regarding whether pDCs are capable of priming and directing $CD4^+$ T cell responses in vivo (Villadangos and Young, 2008). In addition, the application of BST2 mAb in vivo resulted in opposite effects on the generation of $CD4^+$ Teff cells (Swiecki and Colonna, 2010), possibly because of the variation in the types of cells depleted depending on the progression of the immune response. The analysis of Siglec-H-deficient mice, however, indicates that pDCs have the capacity to prime $CD4^+$ T cells for the differentiation of $CD4^+$ Teff cells and $CD4^+Foxp3^+$ iTreg cells through the Siglec-H-mediated regulation of MHC II expression and cytokine production. Alternatively, the results from pDC-ablated mice showed that pDCs are required for the generation of $CD4^+Foxp3^+$ iTreg cells. Taken together, our results support the notion that pDCs promote the induction of peripheral tolerance mediated by the generation of antigen-specific $CD4^+Foxp3^+$ iTreg cells and dampen antigen-specific $CD4^+$ Teff cell responses elicited by cDCs in vivo.

Although the cross-presentation of exogenous antigens in $CD4^-CD8\alpha^+$ cDCs for the priming of $CD8^+$ T cells to elicit CTL immunity has been described (Dudziak et al., 2007; Hildner et al., 2008), the contribution of pDCs to this process in vivo remains under debate (Mouriès et al., 2008; Villadangos and Young, 2008). Although the depletion of pDCs in vivo with BST2 mAb blocked the induction of CTLs (Honda et al., 2005), this might not exclude the involvement of cDCs because they are able to express BST2 after activation (Swiecki and Colonna, 2010). Our findings, however, strongly support the pivotal function in vivo of pDCs as APCs in activating $CD8^+$ T cells through cross-presentation, which is different from the evidence that pDCs are less efficient at cross-presentation than cDCs in vitro. This difference might be explained by the notion that an environmental factor or encountered stimulus prolongs the life span of pDCs and enhances their capacity for cross-presentation. Alternatively, the induction of CTLs by CD40/TLR triggering was reportedly dependent on B7-mediated costimulation and type I IFN in vivo (Ahonen et al., 2004). Furthermore, the interaction between pDCs and cDCs through CD154/CD40 was required for the licensing of cDCs to prime CTL immunity in vitro (Yoneyama et al., 2005). However, the enhanced expression of CD154 on pDCs could be insufficient for the generation of CTLs when there is a deficiency of Siglec-H in vivo. Taken together, pDCs could be required for the efficient priming of $CD8^+$ T cells leading to the generation of CTLs in vivo through the Siglec-H-mediated control of cross-presentation as well as the production of type I IFN and the expression of CD154 for the activation of cDCs.

Published reports suggested that development of $CD4^+Foxp3^+$ nTreg cells is required for interaction of $CD4^+Foxp3^-$ thymocytes with thymic medullary epithelial cells (mTECs) and cDCs (Proietto et al., 2008; Hinterberger et al., 2010). In contrast, our results indicate that pDCs appear to negatively regulate the development of thymic $CD4^+Foxp3^+$ nTreg cells. Thus, pDCs could interfere with the differentiation of thymic $CD4^+Foxp3^+$ nTreg cells by mTECs and cDCs, and Siglec-H-mediated regulation of MHC II expression and cytokine production might contribute to this phenomenon.

cDCs are involved in homeostatic expansion of $CD4^+Foxp3^+$ nTreg cells and generation of $CD4^+Foxp3^+$ iTreg cells in peripheral and mucosal tissues (Kretschmer et al., 2005; Darrasse-Jèze et al., 2009; Fukaya et al., 2010). Our results clearly suggest that pDCs are involved in mucosal homeostasis of $CD4^+Foxp3^+$ nTreg cells, although they are dispensable for peripheral homeostasis of $CD4^+Foxp3^+$ nTreg cells. Furthermore, the mucosal generation of antigen-specific $CD4^+Foxp3^+$ iTreg cells was severely impaired in the absence of pDCs during the oral priming of antigen (data not shown). These observations imply that pDCs participated in de novo generation of $CD4^+Foxp3^+$ iTreg cells in mucosal tissues when they encountered food antigens and commensals under steady-state conditions. Previous studies have suggested that pDCs play a key role in airway tolerance and oral tolerance by inducing the generation of Treg cells as well as deletion and anergy of $CD4^+$ Teff cells (de Heer et al., 2004; Goubier et al., 2008). Therefore, these results suggest that pDCs are crucial for inducing antigen-specific dominant and recessive tolerance to maintain mucosal immune homeostasis.

Mucosal cDCs are suggested to participate in the differentiation of Th17 cells (Darrasse-Jèze et al., 2009; Ohnmacht et al., 2009). Analyses of Th1 cells and Th17 cells in LP of Siglec-H-deficient mice indicate that Siglec-H regulates the capacity of

pDCs to generate $CD4^+$ Teff cells from naive $CD4^+$ T cells. Furthermore, the engagement of type I IFN receptors expressed by DCs reportedly blocked their ability to generate Th17 cells (Shinohara et al., 2008). Thus, inefficient $CD4^+$ T cell priming and robust production of type I IFN by pDCs might explain the impaired generation of LP $CD4^+$ Teff cells under Siglec-H-deficient conditions. More importantly, the results from pDC-ablated mice suggest that pDCs block the differentiation of $CD4^+$ Teff cells through preferential generation and expansion of $CD4^+$ $Foxp3^+$ Treg cells, revealing a pDC-mediated regulatory loop for maintaining immune equilibrium in mucosal tissues.

cDCs have been suggested to control host defenses against intracellular bacterial infections (Jung et al., 2002; Zammit et al., 2005; Neuenhahn et al., 2006). Analysis of pDC-ablated mice suggests that pDCs play a critical role in the initiation and amplification of proinflammatory cytokine production leading to septic shock during early massive bacterial infections. Although cDCs have been described as crucial for inhibiting early bacterial growth (Neuenhahn et al., 2006), dysregulated inflammation reportedly induced a systemic loss of cDCs during bacterial sepsis (Efron et al., 2004). Therefore, the retention of cDCs during the reduced inflammation in the absence of pDCs might explain the effective bacterial clearance. In contrast, pDCs appear to limit antibacterial $CD4^+$ T cell responses, whereas they are required for optimal antibacterial $CD8^+$ T cell responses through the Siglec-H-mediated regulation. It has been suggested that bacterial antigen-specific $CD8^+$ T cells are crucial for eliminating pathogenic microbes (Neuenhahn et al., 2006). Thus, pDCs might contribute to the establishment of the protective immunity against bacterial infections mediated by the cross-priming of antibacterial CTLs. Taken together, our findings indicate the multiple roles of pDCs in the control of inflammation and T cell responses triggered by bacterial infections.

Whereas pDCs have been suggested to be important mediators of host protective immunity against viral infections through their ability to produce type I IFN (Shen and Iwasaki, 2006; Gilliet et al., 2008), how pDCs contribute to the antiviral immune response in vivo remains unclear. A study on the depletion of pDCs and other leukocytes with mAbs to Gr-1 and BST2 suggested a potential helper function of pDCs, aiding the ability of cDCs to prime antiviral CTLs (Yoneyama et al., 2005). Furthermore, a recent study with human blood dendritic cell antigen 2 (BDCA-2)-DTR transgenic mice suggested that pDCs mediate early antiviral type I IFN responses that control viral replication, and this control of viral burden influences the accrual of virus-specific NK or $CD8^+$ T cells (Swiecki et al., 2010). In contrast, analyses of Siglec-H-deficient mice and pDC-ablated mice suggest that pDCs are the main producer of type I IFN, although they make a minimal contribution to proinflammatory cytokine production under the control of Siglec-H during the initial stages of HSV-1 infection in vivo. Furthermore, our results demonstrate the impaired generation of HSV-1-specific CTLs despite the opposite effect on the production of type I IFN. These findings led us to hypothesize that the ability of pDCs to cross-present viral antigen, in addition to their known function as a source of type I IFN, is necessary for the efficient induction of antiviral CTL responses to eliminate viruses.

In conclusion, we described a crucial role of pDCs in the regulation of inflammation and antigen-specific T cell responses in vivo and further showed that Siglec-H provides the fine-tuning of their function. Further elucidation of pDC function might provide critical insight into immune regulation and pathology and aid therapeutic interventions for autoimmune and inflammatory disorders as well as infectious diseases.

EXPERIMENTAL PROCEDURES

Standard methods are described in Supplemental Experimental Procedures.

Mice

The following mice were used in this study: 8- to 12-week-old C57BL/6 mice (Charles River Laboratories), B6.OT-I TCR transgenic mice harboring OVA-specific $CD8^+$ T cells (Dudziak et al., 2007; Hildner et al., 2008), and B6.OT-II TCR transgenic mice harboring OVA-specific $CD4^+$ T cells (Dudziak et al., 2007; Hildner et al., 2008). B6.CD45.1$^+$ OT-I mice and B6.CD45.1$^+$ OT-II mice were bred in-house by crossing B6.OT-I mice and B6.OT-II mice with CD45.1$^+$ B6 mice. *Foxp3*^{EGFP}CD45.1$^+$ OT-II mice were also generated by crossing B6.CD45.1$^+$ OT-II mice with B6.*Foxp3*^{EGFP} mice (Wang et al., 2008). The generation of *Siglech*$^{dtr/dtr}$ mice is detailed in Supplemental Experimental Procedures. The mutant mice were cross-mated for more than nine generations with C57BL/6 mice, and *Siglech*$^{+/+}$ littermates were used as WT mice. All mice were bred and maintained in specific-pathogen-free conditions in the animal facility at RIKEN Research Center for Allergy and Immunology in accordance with institutional guidelines.

In Vivo TLR Stimulation

Mice were intravenously (i.v.) injected with 10 μg of CpG-A oligodeoxynucleotide (ODN) (D19, ggTGCATCGATGCAgggggG; Sigma Aldrich) complexed to 10 μl of in vivo-jetPEI transfection reagent (Polyplus transfection) with or without a peritoneal (i.p.) injection of D-GalN (20 mg/mouse; Sigma-Aldrich). Survival was then monitored for 5 days or sera were collected at the indicated times. In some experiments, Spl was obtained from the mice 24 hr after injection.

Immunization

For the analysis of antigen-specific $CD4^+$T cell responses, mice were immunized subcutaneously (s.c.) with 100 μg of OVA protein (Sigma-Aldrich) emulsified in CFA (Difco), and the Spl was obtained 14 days after the immunization. For the generation of antigen-specific CTLs (Ahonen et al., 2004), mice received an i.v. injection of 10 μg of CpG-A ODN complexed to 10 μl of in vivo-jetPEI transfection reagent in combination with an i.p. injection of 500 μg of OVA protein plus 10 μg of CD40 mAb (clone 1C10; eBioscience), and Spl was obtained from the mice 6 days later.

Statistical Analysis

Data are expressed as the mean ± SD. The statistical significance of the values obtained was evaluated by ANOVA and the Kaplan-Meier log-rank test. A p value of <0.01 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2011.10.014.

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